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PREPARATION OF CAPSAICIN LOADED PLGA MICROSPHERES FOR INJECTION: OPTIMIZATION, CHARACTERIZATION, *IN VITRO* AND *IN VIVO* STUDY

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Abstract

The purpose of this study was to prepare and to characterize a new formulation of injectable capsaicin loaded poly (D,L-lactic-co-glycolic acid) (PLGA) microspheres (CAP-MS). The emulsion solvent evaporation process based on O/W emulsion was applied to prepared the CAP-MS with optimization of formulation using uniform design with three factors and six levels. The optimized formulation was then evaluated in terms of size, encapsulation efficiencies, drug loading, *in vitro* release profile, distribution and pharmacokinetics. According to the mathematic models, the optimal prescription and preparation technology can be conducted at the concentration of PLGA of 2%, the rotation speed of 1000 rpm and the mass ratio (CAP/PLGA) 1:2. The optimized CAP-MS resulted in spherical shapes and possessed a smooth surface. Average diameter, encapsulation efficiency and drug loading were turned out to be 4.73μm, 82.82% and 27.60%, respectively. *In vitro* release study represented a low initial burst release of approximately 21.26% within the first 24 h followed by a prolonged release up to 12 days and the release kinetics fitted well to the Higuchi model. *In vivo* results demonstrated that the drug suspension released rapidly after subcutaneous injection, the accumulate drug release was more than 97% after 12 hours, while the drug loaded microspheres release profile showed a large initial burst effect (59%), and then released slowly, nearly 100% of capsaicin released at the end of 20 days. These results indicate the PLGA microspheres is a promising system that could be exploited as a delivery system for capsaicin with high drug loading capacity and sustained drug release.

Keywords: Capsaicin; PLGA microspheres; Uniform design; Drug release; Pharmacokinetics

1. Introduction

At present, the most commonly used analgesics can be divided into two categories, the most important representatives being: morphine for opioids and aspirin for the non-steroidal anti-inflammatory drugs (NSAIDs), although their clinical effect are better, the adverse reactions are daunting, such as the NSAIDs reaction of gastrointestinal tract, gastric ulcer, stomach bleeding and allergic reaction etc. [1]. Opioid has been restricted because of the potential for addiction and abuse. Therefore, it has been one of the hot spots in the research of new drugs to develop analgesic drugs with high efficiency and low toxicity.

A sort of vanillyl amide alkaloids, capsaicin, which was extracted from natural peppers has shown an extensive physiological pharmacology properties among which the most special are its long-acting analgesic effect and new analgesic mechanism: relieving pain and itching mainly through act on the release, synthesis and storage of neuropeptide substance P, thus capsaicin has been widely used and possess huge potential on application in medicine, with stronger analgesic effect than opioid analgesics and free from the side effects usually caused by NSAIDs[2,3]. Clinically, capsaicin has been used to treat severe post-herpetic neuralgia, sciatica, diabetic neuralgia, rheumatoid arthritis, psoriasis vulgaris and so on [4,5]. Now, however, capsaicin is mainly applied to local administration, such as ointments, cream, patch, membrane, cataplasm, etc, because of its significant first pass effect of hepar [6], excessive short half-life [7] and low oral bioavailability. But all the above-mentioned routes of administration have shown cutaneous stimulation in different degree which limited its clinical application, such as cutaneous thermalgia, local congestion and neurogenic inflammation.

Microspheres of different particle sizes are good adjuvant with many advantages to control release of the encapsulated drugs as well as be functioned in target sites [8]. The microspheres can greatly improve the drug efficacy for its characteristics of masking the bad taste of the medicine, increasing the drug stability and reducing the irritation, toxicity and side effects of the drug [9,10].

Given the disadvantages existed in the course of clinical use, this study aims to develop a biodegradable capsaicin-loaded microsphere with polymers, in order to avoid hepatic first pass effect, reduce its stimulation, maintain drug concentration for a long time, so as to decrease the frequency of administration, reduce its side effects, improve patient compliance, therefore, lay the foundation for further exploration of new form of capsaicin.

2. Materials and methods

2.1 Materials

Capsaicin (CAP) was obtained from Henan Bis Biological Technology Co., Ltd. (Zhengzhou, China). PLGA (lactide: glycolide =75:25, Mw: 10000) was produced by Jinan Daigang Biomaterial Co., Ltd. (Jinan, China). Poly vinylalcohol (PVA, Mw 13,000–23,000) was obtained from Tianjin Tianhe Pharmaceutical Raw Material Factory (Tianjin, China). Gelatin was obtained from Tianjin Fengchuan Chemical Reagent Science And Technology Co., Ltd. (Tianjin, China). CAP acted as an internal standard (IS) for quantitative purposes was acquired from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and Acetonitrile were of HPLC grade and purchased from Fisher Scientific (New York, USA). Distilled water（Wahaha Co., Ltd., Hangzhou, China) was applied throughout the experiment. All other reagents were at least of analytical grade obtained commercially (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

2.2 Preparation of the microspheres

The O/W emulsion/solvent evaporation method was applied to prepare CAP-MS.

Briefly, CAP and PLGA were dissolved in 5.0 ml of methylene chloride under sonication (KQ5200E, Kunshan, China) for 2 min to prepare a drug polymer suspension and then was added to 145ml of aqueous solution containing 0.5% of gelatin under mechanical stirring (1000 rpm) through a syringe. This step was carried out at 25°C in a thermostatic bath (CSY-Ⅱ, Beijing, China). In regard to immobilize the microspheres, a slower stirring at 600 rpm was not stopped until the methylene chloride fully volatilized. The microspheres produced were collected after centrifugation at 3000 rpm for 5 min (LDZ5-2, Beijing, China) and then washed away traces of the residual solvent with distilled water. Finally, the recovered CAP-MS were lyophilized and stored at 4 °C for further study.

2.3 Uniform design

Based on single factor inspection, the experiment for optimization of the formulation parameters of microsphere preparation was arranged as three factors, i.e., the PLGA concentration(X_1), rotation speed (X_2) and the mass ratio (CAP/PLGA) (X_3) by design, each at six levels. The *UD* table $U_6(6^3)$ was used to settle the experiments (Table 1). Each trial was carried out in triplicate. The evaluated response *Y* was the particle size (MD) or drug loading (DLR) or entrapment efficiency (ER).

Table 1 $U_6(6^3)$ uniform design

Level Number	PLGA (%)	Rotation speed (rpm)	m_{CAP} : m_{PLGA} (mg/mg)
1	1 (2%)	2 (600)	3 (1 : 3)
2	2 (2%)	4 (800)	6 (1 : 4)
3	3 (3%)	6 (1000)	2 (1 : 2)
4	4 (3%)	1 (500)	5 (1 : 4)
5	5 (4%)	3 (700)	1 (1 : 2)
6	6 (4%)	5 (900)	4 (1 : 3)

Total synthesis score (Z total), the PLGA concentration(X_1), rotation speed(X_2) and the mass ratio (CAP/PLGA) (X_3) were calculated by Eqs. 1 and 2, in which X was the evaluation index, $\hat{X}$ and S were for the mean and standard deviation of the index respectively; i was the number of index.

$$Z_i = (X_i - \hat{X}_i) / S_i + 5 \quad (1)$$

$$\sum Z_i = \sum Z_{i\max} - \sum Z_{i\min} \quad (2)$$

All trials were performed in triplicate. The Z total, the averages of the MD, DLR and ER were taken as response values. The statistical and linear regression analysis of the data were performed with SPSS16.0 software.

2.4 CAP-MS Characterization

2.4.1 Determination of shape and particle size

The surface morphology of CAP-MS was observed under a FEI ESEMXL-30 scanning electron microscope (SEM) (FEI, Hillsboro, USA). The lyophilized CAP-MS samples were mounted on metal stubs with an adhesive carbon tape, sputter-coated with gold and analyzed with SEM at an acceleration voltage of 15 kV.

The average diameter and size distribution of the lyophilized CAP-MS which were suspended in distilled water were further analyzed by Motic Images Advanced 3.2 software (Motic China Group Co.，Ltd) and the average particle size and D10, D50, D90 were obtained by SAS software, D90 refers to the particle diameter at 90% cumulative size, D10 is the particle diameter at 10% cumulative size and D50 is the particle diameter at 50% cumulative size. For this analysis, the distilled water was used as the dispersion medium.

2.4.2 Determination of CAP loading and encapsulation efficiency

The loading efficiency of CAP encapsulated in the CAP-MS was determined by ultraviolet spectrophotometry method [11]. Briefly, accurately weighed 2.5 g of CAP-MS were dissolved in 10ml of methylene chloride under sonication for 3 in and then the resulting solution were filtered through 0.45μm filter (MillexGV, Millipore, USA). The detection wavelength was set at 280nm. All measurements were carried out in triplicate and the results were given as mean ± SD.

The calculation of DLR (%) and ER (%) were obtained by the following equations, respectively.

DLR (%)=amount of drug in microspheres/ weight of microspheres× 100

ER （%）= actual amount of drug in microspheres/ theoretical amount of drug × 100

2.5 *In vitro* CAP release profile of CAP-MS

The in *vitro* release of CAP-MS was performed in the release medium consisting of phosphate buffered saline (PBS, pH7.4) (sink condition can be maintained) containing 0.05%(w/w) Tween-80 and 0.02% sodium azide and carried out at 37°C. 25mg of CAP-MS were suspended in 1ml PBS and then were poured into a dialysis bag (MWCO 7000) that was immersed in capped glass centrifuge tube containing 500ml PBS. The entire system was laid in a shaker bath (SHZ-82A) and incubated at 37℃ under 75 pm horizontal shaking. Two milliliters of medium was collected from the bag at predetermined time points (1, 2, 3, 5, 7, 10, and 12h) while an equal volume of fresh medium was added in the meantime to maintain a sink condition. The samples gathered were filtered through a 0.45μm filter and then the filtrates were separated for assay by high performance liquid chromatography (HPLC) method.

Briefly, the samples after filtered through the 0.45μm filter were injected into a Shimadzu 1100HPLC system (Shimadzu, Japan). The measurement was carried out on a Shimadzu C18 column (150 x 4.6mm, 5 μm, Shimadzu, Japan) under the column temperature maintained at 30°C by employing a mobile phase comprising methanol: water: phosphoric acid (75:25:0.2, v/v/v) which was delivered at a flow rate of 1.0ml·min^{-1}. And the injection volume was 15μl with the CAP concentrations were detected at 280nm. The quantification and calculations of CAP concentrations were calculated according to the integration of peak areas on the basis of standard curves. The standard curve was A = 9204.9C + 511.63 (r = 0.9998, n=6, the concentration range was 0.63~7.56μg·ml^{-1}) in which A is the peak area and C represents the amount of CAP. All measurements were executed in quadruplicate and the results were expressed as the mean ± SD.

The cumulative release of CAP from the CAP-MS was calculated by dividing the cumulative CAP released into the buffer by the total amount of CAP encapsulated inside the PLGA microspheres (3).

$$Q\% = \frac{C_n \bullet V + V_i \sum_{i=0}^{n=i} C_i}{W \times DL\%} \times 100\% \qquad (3)$$

where C_n is the concentration of CAP at time t, V is the total volume of dissolution medium, V_i is the sample volume at time i, C_i is the concentration of CAP at time i, W is the weight of CAP-MS and $DL\%$ is the drug loading of CAP-MS.

In regard to investigate the mechanism of CAP release from CAP-MS, the kinetic models will be used to analyze the *in vitro* release data, as shown below, zero order (Eq. (4)), first order (Eq. (5)), Higuchi (1963) (Eq. (6)) and Ritger-Peppas[12] (Eq. (7)) equations.

$$Q_t = k_0 t \quad (4)$$

$$\ln(1 - Q_t) = -k_1 t \quad (5)$$

$$Q_t = k_H t^{1/2} \quad (6)$$

$$\ln Q_t = n \ln t + k \quad (7)$$

where Q_t is the cumulative percent fraction of drug release at time t, k is the release rate constant, n is the release exponent which evince the mechanism of drug release. Regression analysis was conducted and the coefficient of determination (R^2) was figured out to appraise the kinetic models. The model which was taken for the most appropriate kinetic model and used to describe the release of CAP from the CAP-MS is the one who gave the highest value of R^2.

2.6 Pharmacokinetic

2.6.1. Animals grouping and drug administration

Pharmacokinetic studies were carried out in healthy female Kunming (KM) Mice weighing 27.2 ± 2.4 g which were supplied by the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). All mice were

acclimatized in animal house for at least 3 days and they received a standard diet and water ad libitum. The mice were fasted overnight before the drug administration but with free access to water throughout the study. The study kept the side of the Guide for the Care and Use of Laboratory Animals.

The mice were randomly divided into two groups receiving pure CAP solution and CAP-MS respectively. Group I received pure CAP solution resolved in saline containing 20mg sodium carboxymethyl cellulose (CMC-Na) per milliliter at a single dose of 24mg·kg^{-1} by subcutaneous injection. Cutting open the epidermis at the sites of injection and cutting out the remaining PLGA microspheres together with the surrounding tissues to get the samples at 0.5, 1, 2, 4, 8, 12, and 24h after dosing. Group Ⅱ received CAP-MS dissolved the same solvent as Group I at the same single dose of 24mg·kg^{-1} by subcutaneous injection and conducted samplings via the same method as Group I at 1, 2, 4, 8, 12h, and 1, 2, 4, 7, 10, 15, 20d after dosing. All the samples were stored at 20°C for analysis.

2.6.2 Preparation and quantification of samples

The CAP concentration in samples was detected by the HPLC method as described above with the standard curve (A = 8493.5C + 1291, r = 0.9993, n=7, 0.1~30 μg·ml^{-1} concentration range). The cut-up samples were impregnated with 1 ml of saline and mixed for 10 min after thawing at room temperature and then transferred the sample into 50ml volumetric flask. Adding 9 ml of acetonitrile in several times to wash the residues under sonication for 10 min followed by the addition of PBS containing 0.08% (w/w) Tween-80 to the scale line with vortexing for 2 min and then centrifuged the entire system at 3000 rpm for 10 min. The supernatant was filtered through a 0.45 μm filter and was injected into the HPLC system for analysis. All measurements were carried out in triplicate and the results were expressed as the mean ± SD.

The release capacities of CAP from CAP-MS were calculated by Eqs. 7 in which C_r was the release capacities of CAP from CAP-MS, C_t was the total CAP content in CAP-MS and C_s was the concentration of CAP in the samples.

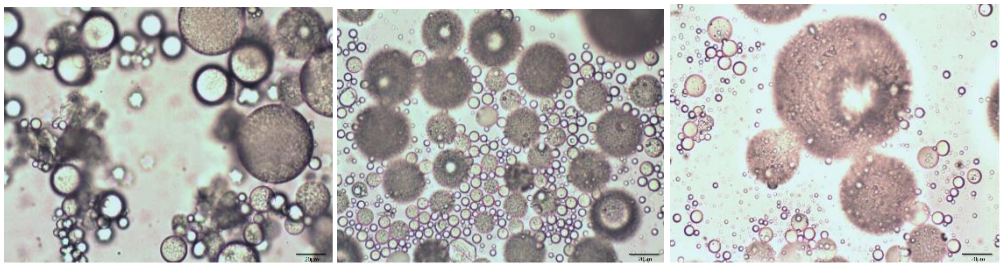
$$C_r = C_t - C_s \quad (7)$$

3. Results and discussion

3.1. Impact of four formulation parameters on the ER of CAP-MS

CAP-MS have been successfully fabricated by O/W emulsion/solvent evaporation method. In contemplation of the demand of getting high DLR and the reality that CAP has very poor aqueous solubility, the CAP-MS were prepared by O/W emulsion/solvent evaporation method [13,14].

This study investigated the outcome of variation in four key formulation parameters that were screened by the single factor experiment [15], as illustrated in Table 2 to Table 5 and Figure 1 to Figure 4, on the physicochemical characteristics of CAP-MS. The dispersion phase type and its concentration were investigated. The concentration of PLGA was also examined, together with the preparation temperature. Experimental responses, such as particle size, morphology, and encapsulation efficiency were determined.

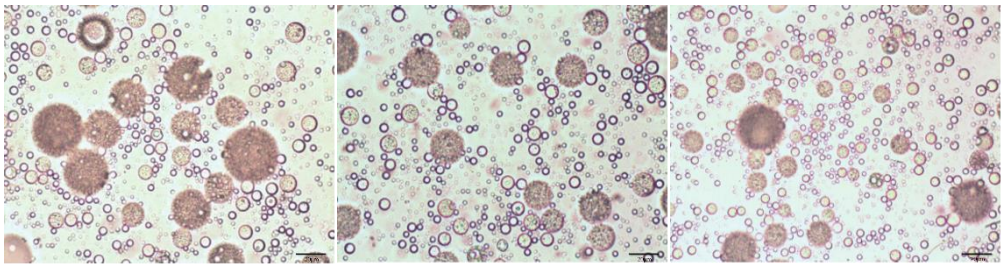


A1 A2 A3

Figure 1. Microspheres prepared with varied dispersing agents (×400). (A1) the dispersion phase is PVA; (A2) the dispersion phase is gelatin; (A3) the dispersion phase is Tween-80

Table 2. Influence of varied dispersing agents on the preparation of microspheres

Number	Dispersing agents	Microspheres	Encapsulation rate %
A1	PVA	spherical, regular, aggregate, wide particle size distribution	63.62
A2	Gelatin	spherical, regular, good dispersion, narrow particle size distribution	88.09
A3	Tween80	spherical, regular, good dispersion, wide particle size distribution	0

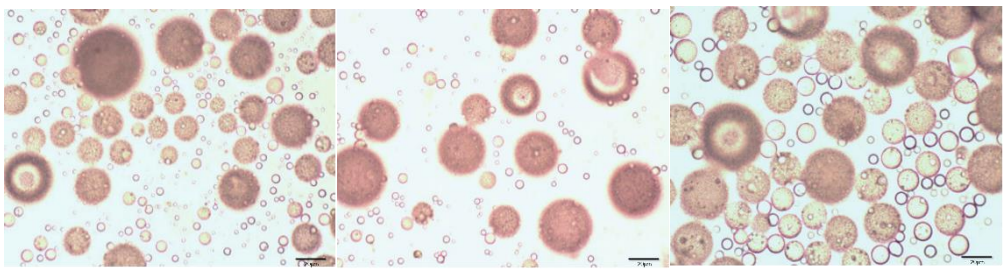


A4 **A5** **A6**

Figure 2. Microspheres prepared with varied gelatin concentrations (×400). (A4) the concentration of gelatin is 0.5%; (A5) the concentration of gelatin is 1.5%; (A6) the concentration of gelatin is 3.0%.

Table 3. Influence of varied gelatin concentrations on the preparation of microspheres

Number	Gelatin (%)	Mean diameter (μm)	Drug-loading rate (%)	Encapsulation rate (%)	Yield (%)
A4	0.5	5.56	15.98	79.91	83.60
A5	1.5	3.27	14.01	70.33	75.30
A6	3.0	3.12	10.10	50.69	64.94



A7 **A8** **A9**

Figure 3. Microspheres prepared at varied temperatures (×400). (A7) the preparation temperatures is 20℃; (A8) the preparation temperatures is 25℃; (A9) the preparation temperatures is 30℃. The particle size gradually increased.

Table 4. Influence of temperatures on the preparation of microspheres

Number	Temperature (℃)	Mean diameter (μm)	Drug-loading rate (%)	Encapsulation rate (%)	Yield (%)
A7	20	5.89	14.06	70.29	84.00
A8	25	6.33	14.81	74.37	78.09
A9	30	12.86	12.29	61.47	75.20

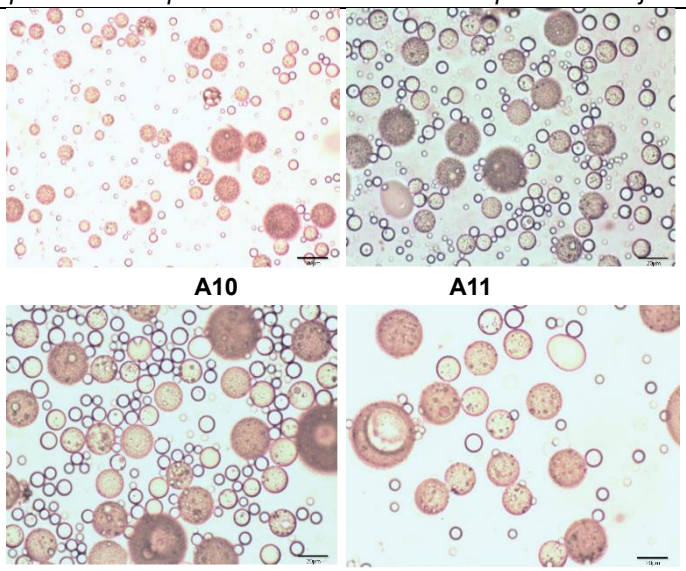


Figure 4. Microspheres prepared with varied concentrations of PLGA (×400). (A10) the concentrations of PLGA is 2%; (A11) the concentrations of PLGA is 3%; (A12) the concentrations of PLGA is 4%; (A13) the concentrations of PLGA is 5%.

Table 5. Influence of varied concentrations of PLGA on the preparation of microspheres

Number	PLGA （%）	Mean diameter （μm）	Drug-loading rate （%）	Encapsulation rate （%）	Yield （%）
A10	2 (100mg)	4.89	27.67	83.56	8.13
A11	3 (150mg)	6.59	19.44	78.16	83.58
A12	4 (200mg)	6.96	15.89	79.78	76.49
A13	5 (250mg)	13.55	13.01	78.83	73.93

The dispersion phase type has discernible effect on the preparation of CAP-MS (Figure 1 and Table 2). When the dispersion phase is PVA or Tween80, the particle size was widely distributed whereas the gelatin made the narrow distribution of particle size. Moreover, when the dispersion phase is PVA, the microspheres distributed as clump and when the dispersion phase is Tween-80, the encapsulation efficiency is 0, while the gelatin will not cause these situations. The MD, DLR (%) and ER (%) gradually decreased with the increase of the concentration of gelatin (Figure 2 and Table 3). the preparation temperature has been demonstrated as a key parameter influencing the properties of the resultant microspheres. As shown in Figure 3 Table 4, when the preparation temperature increased, the particle size gradually increased, but the DLR (%) and ER (%) increased first, then decreased. What's more, the particle size gradually increased, but the drug loading (%) and encapsulation efficiency (%) gradually decreased with the increase of the concentration of PLGA (Figure 4 and Table 5).

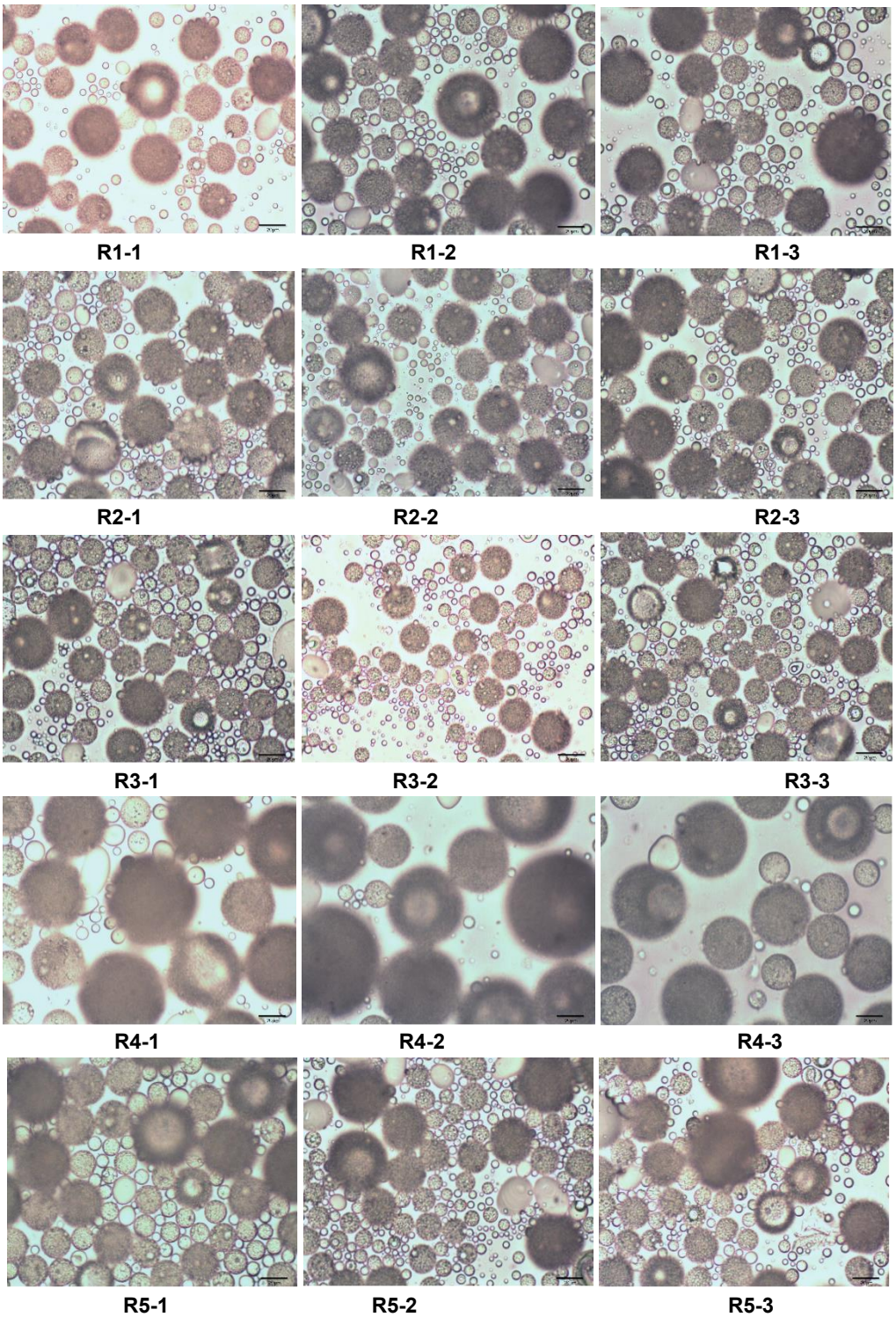
3.2 Uniform design

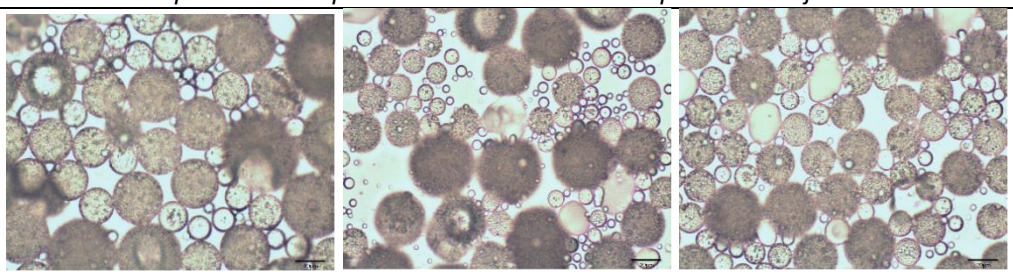
3.2.1 Statistical analysis

Uniform design was utilized to select the optimal prescription and preparation technology which is suitable for selecting factors and for constructing linear regression models using uniform-design-table, thus allowing to optimize the process with a few of experimental runs

[16,17]. The design of the experiments and the results are displayed in Figure 5 and Table 6. The equation relating the coefficients obtained for the MD, the DLR, ER and total synthesis score (ZS) to the experimental variable is as shown below and the analysis of variance for the mathematical models was listed in Table 7.

$$Y_{MD}= 16.109+533.036X_1-0.012X_2-27.981X_3$$
$$Y_{DLR}=-.290+201.504X_1+0.001X_2+52.428X_3$$
$$Y_{ER}=40.810+807.289X_1+0.003X_2+34.377X_3$$
$$Y_{ZS}=-3.060-5.028X_1+0.003X_2+16.733X_3$$





R6-1 R6-2 R6-3

Figure 5. Microspheres of the test numbers（×400）

Table 6. The results of uniform design

Test Number	Mean diameter μm	Drug-loading rate %	Encapsulation rate %	Z-Score
R1-1	9.16	17.84	71.91	4.54
R1-2	12.33	18.13	73.06	4.04
R1-3	10.78	17.22	69.42	3.89
R2-1	9.5	14.8	73.98	4.21
R2-2	9.3	11.78	58.88	2.4
R2-3	7.94	13.34	66.71	3.64
R3-1	8.51	28.57	85.71	7.55
R3-2	6.06	28.61	85.82	8.07
R3-3	6.47	28.83	86.48	8.08
R4-1	20.7	13.69	67.71	1.16
R4-2	20.2	17.94	88.76	3.85
R4-3	18.56	14.2	70.28	1.91
R5-1	16.33	29.84	89.53	6.48
R5-2	13.19	31.29	93.87	7.74
R5-3	12.19	30.2	90.59	7.49
R6-1	18.09	21.39	85.23	4.46
R6-2	16.77	23.58	93.96	5.87
R6-3	17.81	21.56	85.91	4.61

Table 7. Multiple linear regression

Dependent variable	Regression equation	R	F	P
Mean diameter	YMD=16.109+533.036X1-0.012X2-27.981X3	0.953	46.36	1.61E-07
Drug-loading rate	YDLR=-4.290+201.504X1+0.001X2+52.428X3	0.987	173.50	2.63E-11
Encapsulation rate	YER=40.810+807.289X1+0.003X2+34.377X3	0.875	15.18	1.11E-04
Z-Score	YZS=-3.060-5.028X1+0.003X2+16.733X3	0.947	40.20	3.95E-07

The mathematical model was expressed as the means of the values of all the independent variables and by ignoring the statistically negligible terms. As the value of R is close to 1, the correlation between the experimental and the predicted values are better [18]. The values of R were 0.953 for mean diameter, 0.987 for drug-loading rate and 0.875 for entrapment rate, which demonstrated that the obtained data coincide well with predicted values.

In general, the model which the calculated *F* is greater than that of the critical value is a well prediction for experimental results. It was demonstrated that all the equations in this work for which the computed *F* values were greater than that of the critical values and the regression coefficients were statistically significant were authentic (Table 7).

3.2.2 Optimization and model validation

It is well known that the MD and ER are the 2 key physicochemical properties of microspheres [19,20]. The ER is vital for evaluating the drug loading capability of microspheres. Therefore, the high ER is helpful for reducing the loss of drug and extending the duration and dosage of treatment [21]. As for the CAP-MS, the smaller the MD and the higher the DLR and ER, the better the quality will be. However, there is a contradiction between the optimal conditions of MD, DLR and ER (Table 6), the indexes were normalized with the way of Z-Score comprehension evaluation. The optimal formulation and preparation process was subsequently obtained: the concentration of PLGA is 2%, the rotation speed is 1000 rpm and the mass ratio (CAP/PLGA) is 1:2.

To confirm the validity of the model, six batches of microspheres (Y1~Y6) were prepared by the optimum preparation technology. Pluging the new data into the regression equations to get the predicted value $\hat{Y}$ which interval estimate is $Y = \hat{Y} \pm U_{\alpha(0.05)} \bullet S$ (Table 8). The result indicated that the actual value agrees the predicted value (Table 9). Hence, the microspheres prepared by the optimized formulation were used in follow-up experiments.

Table 8. Prediction of results of the optimum prescription

Index	Regression equation	Estimate	Standard error	Predictive value, 95%	
				L	U
Mean diameter	Y_{MD}=16.109+533.036X1-0.012X2-27.981X3	0.78	1.62	-2.39	3.95
Drug-loading rate	Y_{DLR}=-4.290+201.504X1+0.001X2+52.428X3	26.95	1.20	24.60	29.31
Encapsulation rate	Y_{ER}=40.810+807.289X1+0.003X2+34.377X3	77.14	5.75	65.88	88.41
Z-Score	Y_{ZS}=-3.060-5.028X1+0.003X2+16.733X3	8.21	0.77	6.70	9.71

Table 9. The actual results of the optimum prescription

Number	Mean diameter (μm)	Drug-loading rate (%)	Encapsulation rate (%)	Z-Score
Y1	6.45	29.35	88.05	8.31
Y2	3.64	25.72	77.16	7.34
Y3	4.07	27.93	83.80	8.19
Y4	5.12	28.50	85.51	8.22
Y5	5.28	28.55	85.66	8.21
Y6	3.84	25.57	76.71	7.23

3.3 Characterization of CAP-MS

The surface morphology of CAP-MS observed by the SEM is shown in Figures 6 and 7, which illustrated that the CAP-MS were globosity in shape with a smooth surface.

As shown in Figure 8, the MD of the CAP-MS was 4.73μm and the D10=0.72, D50=1.94, D90=8.52.

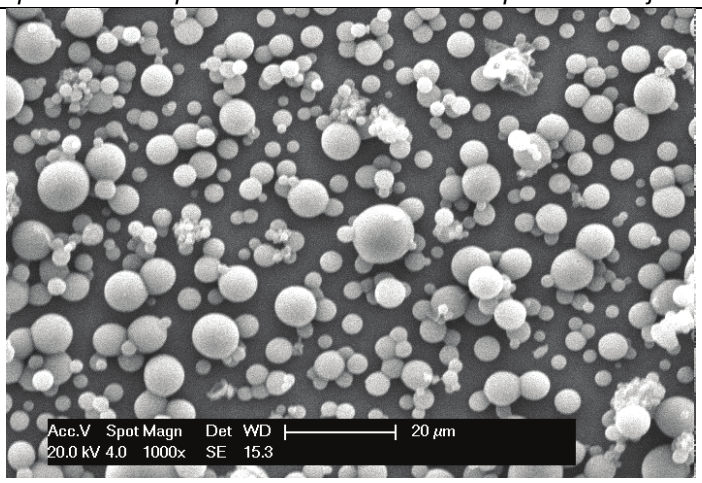


Figure 6. SEM photography of blank PLGA microspheres

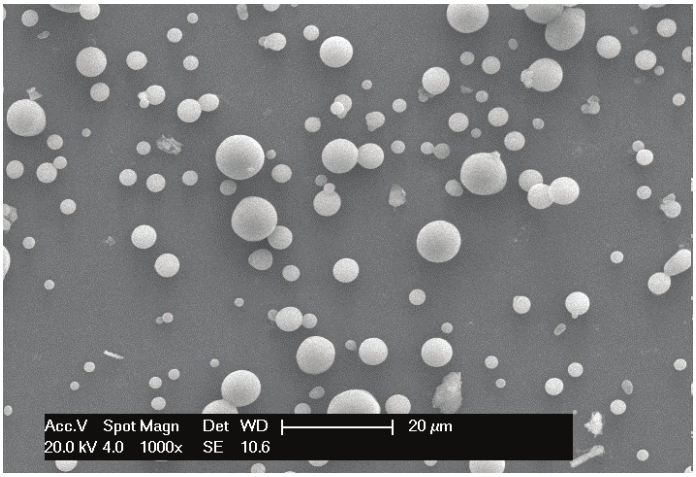


Figure 7. SEM photography of CAP-loaded PLGA microspheres

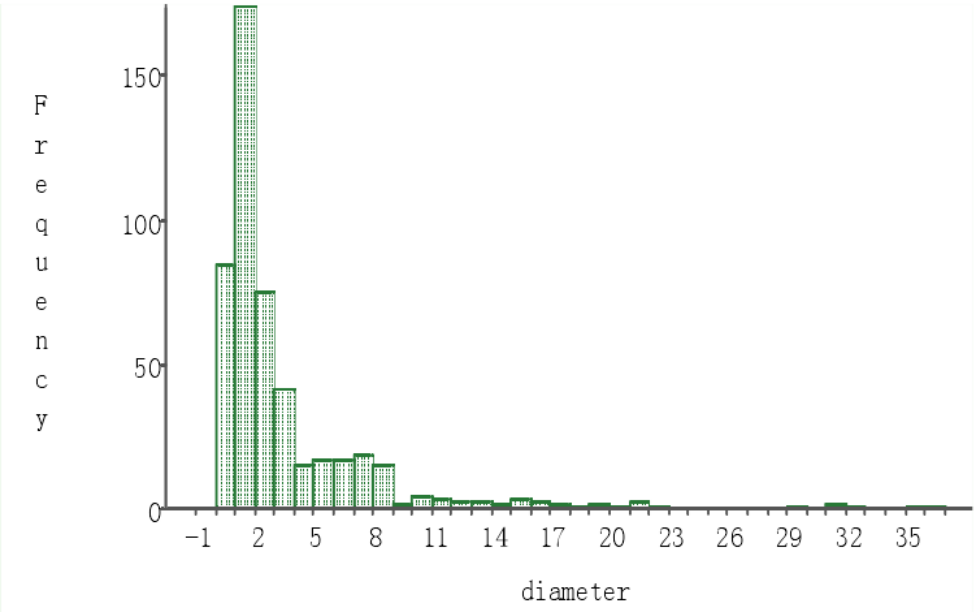


Figure 8. The particle diameter distribution of the optimum prescription

The ER and DLR of the CAP-MS produced with optimum prescription were determined to be 82.82% and 27.60, respectively.

3.4 *In vitro* CAP release studies

The cumulative release profile of CAP from the CAP-MS in release medium is shown in Figure 9 and Table 10. The result exhibited a sustained release of CAP from the CAP-MS up to 12 days after a low initial burst release of about 21.26% within the first 24 h which is likely to be the release of CAP on the surface of microspheres, and the next subsequent release phase is the result of the diffusion of the drug and the dissolution of the matrix.

The PBS which pH is 7.4 similar to the organizational environment is often used as the dissolution medium for sustained-release injectable microspheres [22]. In this study, due to the long release period of the microspheres, 0.02% NaN_3 was added as an antibacterial agent. In consideration of the low water solubility of capsaicin, 0.05% of Tween80 was added to improve the solubility of capsaicin in aqueous medium so as to meet the sink condition. The addition of Tween80 can significantly increase the solubility of capsaicin（65.80 μg·ml^{-1}）, and the solubility of capsaicin would be much better (129.47 μg·ml^{-1}) with the addition of NaN_3 at the same time which may be related to the chemical structure of capsaicin.

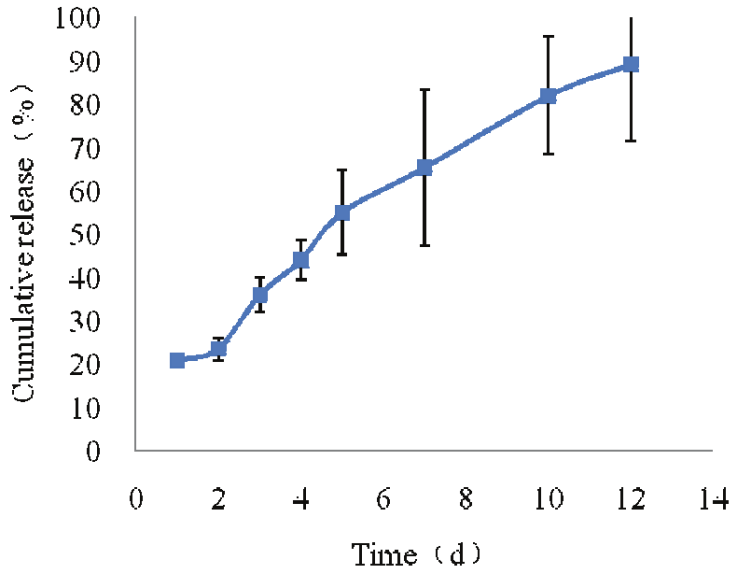


Figure 9. The release curve of CAP-PLGA microspheres *in vitro* at 37℃

Table 10 Cumulative release of CAP in PLGA microspheres in vitro（37℃，n=4）

Time (d)	Cumulative release (%)
1	21.06±1.34
2	23.65±2.57
3	36.06±3.91
4	44.14±4.53
5	55.10±9.83
7	65.46±17.93
10	81.99±13.54
12	89.34±17.75

The mechanism of CAP release from the CAP-MS was studied by fitting the data obtained from *in vitro* release study into the kinetic models mentioned above. The coefficient of determination (R^2), release rate constant (k) and n values acquired after linear regression on various kinetic models are displayed in Table 10. The results showed that the *in vitro* release data was followed and supported by the Higuchi model owing to the maximum of R^2 (0.987) it presented. The $t_{1/2}$ was 109.73 h which suggests the CAP-MS has remarkably sustained release action.

According to the Ritger-Peppas equation $Q = kt^n$, the releasing indexes (n) was 0.639 which suggested that the drug release was non-Fick diffusion determined, the mechanism may be involved: the dissolution/diffusion of drug from the matrices, and the stroma erosion in virtue of degradation/dissolution of PLGA.

3.5 *In vivo* pharmacokinetic study

The percent of residual drug and in vivo drug release percentage of CAP in mice after hypodermic injection administration of CAP-MS and capsaicin suspension are shown in Table 12 and Table 13 and the cumulative release curve is shown in Figure 10. The results demonstrated that the drug suspension released rapidly after subcutaneous injection, the accumulate drug release was more than 97% after 12 hours, while the drug loaded microspheres release profiles showed a large initial burst effect (59%), and then released slowly, nearly 100% of CAP released after 20 days.

Table 11. The regression equation of CAP release *in vitro*（37℃）

Mode	Regression equation	R^2
Zero order	$Q = 0.003t + 0.165$	0.974
First order	$\ln(1-Q) = 0.091 - 0.008t$	0.984
Higuchi	$Q = -0.139 + 0.061t^{1/2}$	0.987
Ritger-Peppas	$\ln Q = 0.639 \ln t - 3.723$	0.970

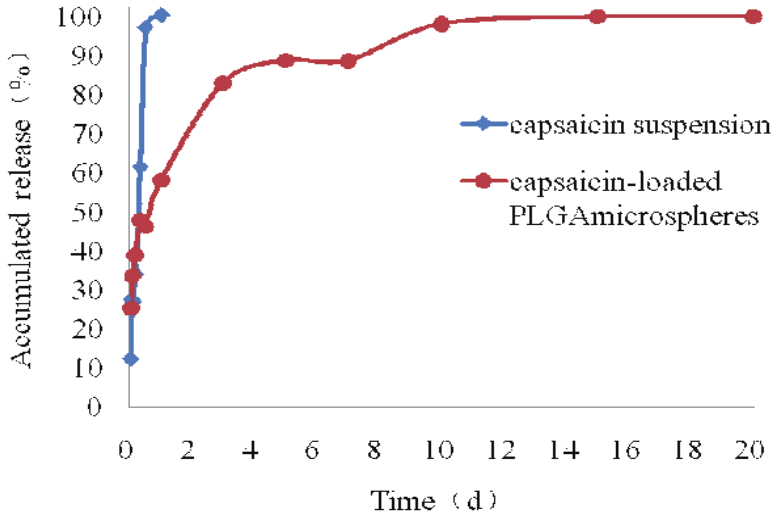


Figure 10. Drug release curve of CAP in mice in vivo（n=3）

Table 12. Residual percent and accumulative release percent of CAP-loaded PLGA microspheres in mice (n = 3)

Time (d)	Residual drug (%)	Accumulative release (%)
0.04	72.97±11.07	27.03
0.08	65.00±16.59	35.00
0.17	59.77±7.10	40.23
0.33	51.02±10.84	48.98
0.50	52.39±4.27	47.61
1.00	41.00±0.60	59.00
3.00	16.61±2.12	83.39
5.00	11.04±0.12	88.96
7.00	11.09±1.80	88.91
10.00	1.88±0.58	98.12
15.00	0.08±0.01	99.92
20.00	0.03±0.01	99.97

Table 13. Residual percent and accumulative release percent of CAP suspension in mice (n=3)

Time (h)	Residual drug (%)	Accumulative release (%)
0.5	87.72±7.62	12.28
1	72.50±16.53	27.50
2	73.09±8.45	26.91
4	65.87±7.50	34.13
8	38.44±17.23	61.56
12	2.61±1.70	97.39
24	0	100.00

3.6 *In vitro–in vivo* correlation

The *in vitro* and *in vivo* correlation is continuous to be an essential issue for the dosage forms in which the drug needs to be absorbed into circulation, on account of the importance for both formulation exploration and quality control [23]. Through the comparison of the two data in this research, it was found that the drug release rate was higher than that in vitro that may cause by the different mechanism of PLGA degradation which is due to the presence of enzymes and other factors such as intercellular fluid volume and the local pH. The degradation of PLGA microspheres *in vivo* was initiated from the surface, while some of the PLGA microspheres were degraded *in vitro* from inside to outside, resulting in the degradation process of the acidic oligomers [24,25].

4. Conclusion

In the present study, the injectable PLGA microspheres were used to develop a sustained-release delivery system of CAP to extend the clinical application. CAP-MS have been prepared using the solvent evaporation method based on O/W emulsion and an optimization strategy, the

preparation process is stable, this formulation endowed with good DLR and high ER, and the microspheres turned out to be globular shapes, smooth surface, with good dispersion and was narrow in size distribution. The microspheres represented a prolonged duration of release with regard to suspension when administrated via subcutaneous injection, however, with a large initial burst. The microspheres could be stored up for at least 5 months under seal at room temperature, keeping away from light. Therefore, these results suggest that the PLGA microspheres might be a promising formulation for CAP used in clinic.

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